5.3

Reactivation of Latent Viruses

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ABSTRACT

Reactivation of latent viruses may pose an important health risk for people living and working in extreme environments, such as space and Antarctica. Stress-induced changes in immune function under such conditions may increase the incidence and duration of viral reactivation and shedding. We studied viral shedding and reactivation patterns in 8 subjects (5 male and 3 female) participating in 60-day (Phase IIa) and 91-day (Phase III) chamber studies of the Lunar-Mars Life Support Test Project (LMLSTP) at NASA Johnson Space Center. Saliva, blood, and urine samples were collected from the 8 subjects before, during, and after chamber isolation. Using a polymerase chain reaction assay, saliva samples were processed for Epstein-Barr virus (EBV) DNA and urine samples were analyzed for cytomegalovirus (CMV) DNA detection. EBV DNA was detected in 35% of the total saliva samples from both chamber studies, 13% (range 0 to 31%) of samples from the 60-day study, and 51% (range 33 to 81%) of the samples from the 91-day study. Detection frequency was highest prior to chamber isolation. Although CMV DNA was not detected in any of the urine samples collected from these subjects before and after the studies, antibody titers to CMV were significantly increased over control values. Interestingly, EBV antibody titers did not differ significantly from the controls. Our current finding of increased viral reactivation in closed-chamber study subjects agrees with our previous Space Shuttle and Antarctic data. These results support and extend our previous observations that latent viral reactivation increases during space flight, which is consistent with a stress-induced decrease in immune function.

Introduction

Herpes viruses are the most readily recognized latent viruses and the leading infectious cause of blindness in the United States (10). The establishment of viral latency and subsequent reactivation are not well understood, but decreased cellular immunity is known to increase the incidence and duration of reactivation and shedding of some latent viruses (1, 2). Reactivation of latent viruses may cause no symptoms or may produce significant illness (e.g., shingles). These viruses are

carried by astronauts into space and may pose an important health risk in a closed-loop environment. The risk of reactivation almost certainly increases as the duration of space missions increases. Risks associated with many infectious agents are reduced by the preventive measures of the health stabilization program before flight. However, reactivation of latent viruses is unaffected by such actions. A variety of stress factors may cause reactivation of these viruses, such as psychosocial stress, trauma, sunlight, respiratory infection, and fever.

Epstein-Barr virus (EBV), a DNA virus, infects more than 90% of the adult population worldwide and is the etiologic agent of infectious mononucleosis (4, 9). EBV is highly infectious and can be transmitted by microdroplets and by direct contact with saliva. Normally, primary infection is a self-limiting disease leading to a lifelong persistence of EBV in the B lymphocytes or epithelial cells of the oropharynx of infected individuals. EBV is also associated with serious illnesses including Burkitt's lymphoma, nasopharyngeal carcinoma, diffuse oligoclonal B-cell lymphoma, Hodgkin's disease, AIDS-associated lymphoma, and post-transplant lymphoproliferative disease. EBV can be found in saliva after reactivation, which can be triggered by a variety of factors, perhaps including space flight. The physical and psychological stresses associated with the launch and landing of spacecraft, and living and working in the crowded, closed environment in microgravity, may result in viral reactivation and shedding. The effects of stress on viral reactivation are probably mediated through the endocrine-immune axis (1, 2, 3, 5).

Numerous studies have shown altered immune function among individuals living and working together in space and in selected ground-based analogs of space flight. These alterations include phenotypic changes within leukocyte populations, impaired neutrophil function, decreased natural killer (NK) cell activity (15), reduced T-cell proliferation to mitogenic stimulation, altered cytokine production (13), and depressed cutaneous delayed-type hypersensitivity response (12, 16). Reduction in cell-mediated immunity (CMI) may lead to viral reactivation, which may be expressed as a) asymptomatic viral shedding, b) a localized clinical infection (e.g., herpetic lip lesion) limited by the cell-mediated immune system to a cell-to-cell transfer of virus, or c) a generalized local infection or a more severe disseminated infection.

Previously, we investigated latent EBV as a candidate virus for latent viral reactivation and reported a greater frequency of EBV DNA shedding by astronauts before space flight than during and after flight (11). Reactivation of EBV occurred before flight, and eight-fold or greater increases in anti-EBV antibodies were observed during flight (14). Recently, in a ground-based space analog study conducted in Antarctica, we reported increased salivary EBV shedding with diminished CMI response during long periods of isolation (7). The results of these studies suggested that decreased cellular immune function leads to reactivation and shedding of potentially infectious viruses. Thus, a major concern associated with space flight-induced immunosuppression is the possibility of infectious diseases posing an unacceptable medical risk to subjects.

The limited access to space requires the use of ground-based analogs of space flight. Environmental chambers have been used since the Skylab program (1973-1974) to simulate specific aspects of space flight. In the present study, the polymerase chain reaction (PCR) was used to identify EBV DNA in saliva and CMV DNA in urine as a measure of viral shedding in response to 60-day (Phase IIa) and 91-day (Phase III) chamber studies of the Lunar-Mars Life Support Test Project (LMLSTP) at NASA Johnson Space Center.

Materials and Methods

Subjects

Eight subjects (5 males and 3 females) participated in the two chamber studies, one of 60 days and the other of 91 days (4 subjects each). Their ages ranged between 28 and 42 years. The control group was composed of 11 healthy age-matched adults (10 males and 1 female).

Samples

Saliva, blood, and urine were collected from each subject participating in the LMLSTP Phase III study. As shown in Table 5.3-1, saliva was collected every other day upon arising, and blood and urine were collected once before and once after the study. Saliva samples were processed for EBV DNA, whereas urine samples were processed for CMV DNA detection.

Table 5.3-1 *Schedule of sample collection from subjects during the 60-day and 91-day chamber studies*

Sample	Study Phase			
	Prechamber		In-Chamber	Postchamber
Saliva (2-3 ml)	60-day study	MWF for 2 weeks immediately before entry	MWF	MWF for 2 weeks immediately after exit
	91-day study	MWF for 4 weeks beginning 8 weeks before entry	MWF	MWF for 2 weeks immediately after exit
Blood (10 ml)	Once, immediately before entry		None	Once, immediately after exit
Urine (3 ml)	Once, immediately before entry		None	Once, immediately after exit

MWF = Monday, Wednesday, and Friday

Saliva samples were collected with Salivette kits (Sarstedt, Inc., Newton, NC), which consist of a cotton roll in a polypropylene vial. Subjects placed the roll in their mouth until it became saturated, and then returned the roll to the vial. Saliva samples were centrifuged immediately after collection and stored frozen at -70°C. All samples collected from a given subject were analyzed simultaneously. Saliva (2 to 3 ml) and urine (3 ml) specimens were concentrated with a 100-KD filtration unit (Filtron Technology Corp., Northborough, MA). DNA was extracted by a nonorganic extraction method (Qiagen Inc., Chatsworth, CA), and EBV DNA was detected as described earlier (6, 11) and described here briefly as follows. The PCR primers were directed at the EBV polymerase accessory protein gene (BMRF1): P1, 5'-GTC CAA GAG CCA CCA CAC CTG (The Midland Certified Reagent Co., Midland, TX), and P2, 5'-biotin CCC AGA AGT ATA CGT GGT GAC GTA GA (Digene Diagnostics, Gaithersburg, MD). These primers were used at a concentration of 200 µM with 10 µM deoxynucleic acid triphosphates (Perkin Elmer, Branchburg, NJ). PCR was optimized using buffer II (Perkin Elmer) with 2.5 mM $MgCl_2$. DMSO (Sigma, St. Louis, MO) was added to a final concentration of 5%. AmpliGold (2.5 units per 100 µl reaction mixture) (Perkin Elmer) was added, and 5 µl of the purified DNA was added to 20 µl of the reaction mixture. The cycle parameters were 95°C for 9 min, followed by 40 cycles of 94°C for 15 sec, 61°C for 15 sec, and 72°C for 15 sec, with a final extension step at 72°C for 5 min.

CMV DNA was detected using primers that target the major immediate early gene (P1, 5'-TGT CCT CCC GCT CCT C, and P2, biotin 5'-ATG AAG GTC TTT GCC CAG TA). All reactions were carried out using a Perkin-Elmer GenAmp system 9600. An initial denaturation step of 94°C for 9 min was followed by 40 cycles of 94°C for 30 sec, 69°C for 15 sec, 72°C for 30 sec, 72°C for 7 min, and a 4°C hold. The amplified product was analyzed for the presence of EBV or CMV using the Digene Sharp Signal System (Digene Diagnostics Inc., Gaithersburg, MD) according to the manufacturer's instructions.

Measurement of Antiviral Antibody Titers

EBV and CMV antibody titers were determined by indirect immunofluorescence assay. Commercially prepared substrate slides and control sera were used for determining antibody (IgG) titers for viral capsid antigen (VCA) and early antigen (EA) of EBV, CMV, and measles (Bion Enterprises, Park Ridge, IL). Four-fold dilutions of plasma were prepared with phosphate-buffered saline. The endpoint titer was determined as the highest dilution of serum in which immunofluorescent-positive cells could be detected. All specimens were batch analyzed and read blind-coded.

Samples from 11 healthy age-matched adults were collected as controls. Saliva, urine, and blood samples (one of each) were collected from each of these subjects on day 1, day 7, and day 22 of the tests. This collection schedule closely approximates the pre- and postflight collection schedule of a 12-day Shuttle flight. One-time urine samples were also collected from 30 additional healthy age-matched subjects for CMV analysis.

Data Analysis

The frequency of viral shedding in saliva and viral antibody titers in plasma from chamber study subjects and controls were tested for normality. One-way analysis of variance was performed to study significant differences across different times during the study.

The method of generalized estimating equations with a logit link was used to find significant difference between the phases (pre-, in-, and postchamber). The differences between sampling periods were considered significant if $P < 0.05$.

Findings

A total of 418 saliva samples were collected from the eight subjects participating in the two chamber studies. These samples were analyzed for EBV DNA using PCR.

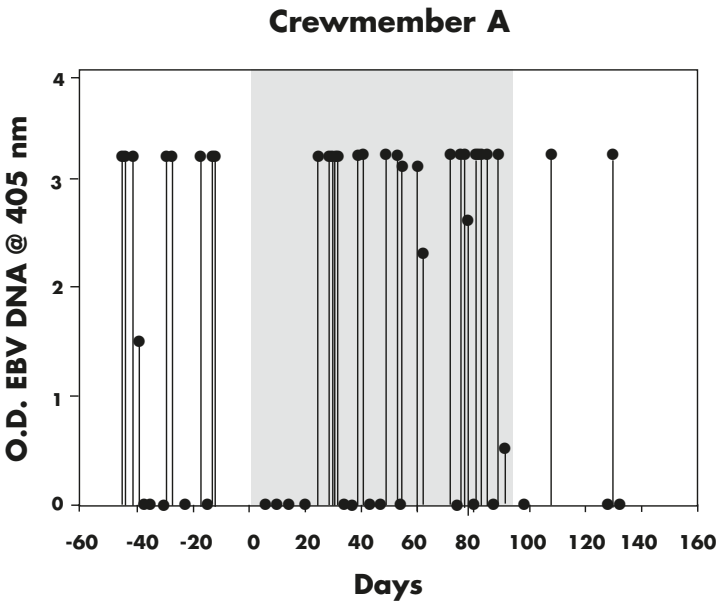
Overall, EBV DNA was detected in 35% of the 418 total saliva samples collected from these subjects. The subjects in the 60-day chamber study had EBV DNA in 13% (range 0 to 31%) of their samples, and the subjects in the 91-day study

Table 5.3-2 *EBV DNA presence in saliva from chamber subjects*

	Crewmember	Saliva Samples		
		Number of Samples	Number of Samples Positive for EBV DNA	% Positive
91-Day Chamber Study	A	62	50	81
	B	56	32	57
	C	67	22	33
	D	62	22	35
60-Day Chamber Study	E	48	15	31
	F	42	2	5
	G	41	5	12
	H	40	0	0
TOTAL	8	418	148	35
Control	11	27	1	4

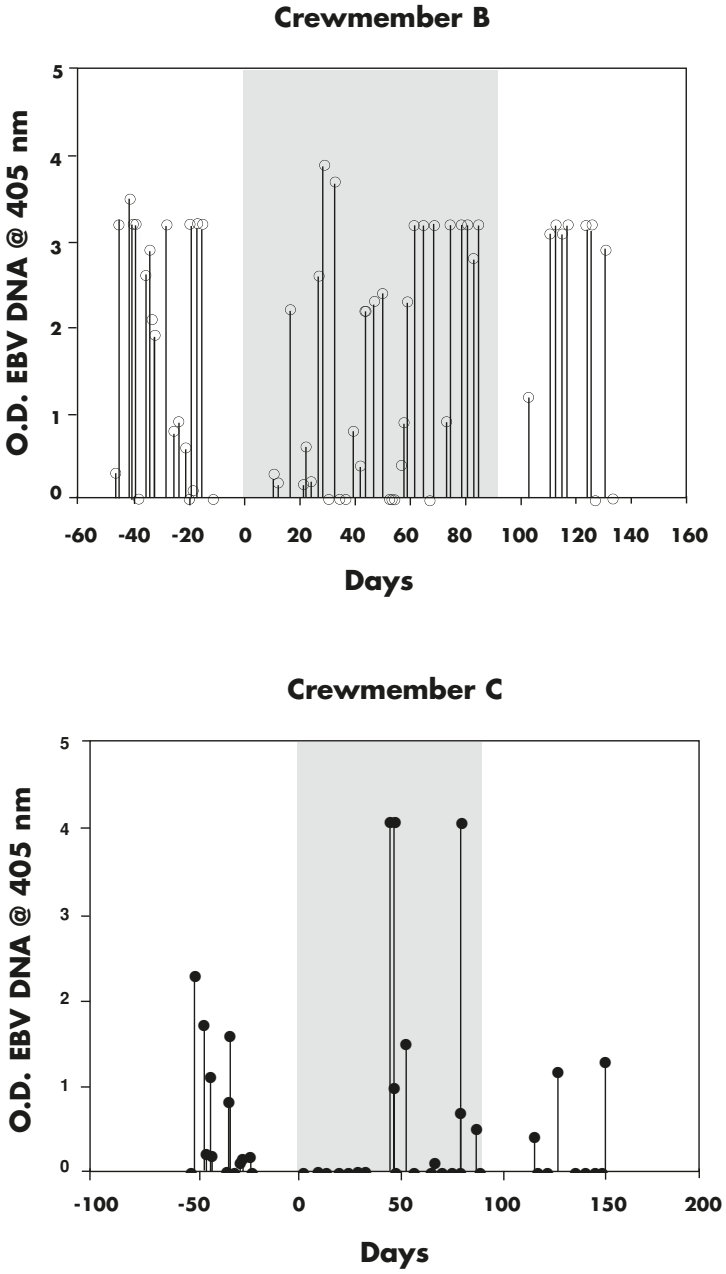
had EBV DNA in 51% (range 33 to 81%) of their samples (see Table 5.3-2). The individual EBV DNA shedding patterns are shown in Figure 5.3-1. During both studies, all but one subject (in the 60-day chamber study) shed EBV. Subject H did not shed EBV in 40 saliva specimens collected over the 88-day collection period. EBV shedding frequency by two of these subjects (A and B of the 91-day chamber study) was very high (57% and 81% EBV-positive saliva specimens), while the other 6 subjects had low to moderate shedding frequencies (see Table 5.3-2). The average shedding frequency of EBV in saliva samples (collected before, during, and after chamber) was significantly greater for the chamber subjects (35%) than

the control group (4%) ($P < 0.005$). Although a higher shedding rate (51%) was observed in the 91-day study than in the 60-day study (13%), the difference between the shedding frequencies was not statistically significant. Subsequent analyses were, therefore, performed on the pooled data from both studies. The incidence of positive EBV findings for the chamber subjects and the control subjects was compared across the three phases (prechamber, in-chamber, and postchamber). The incidence rates for the three phases were determined as follows: prechamber, 37%; in-chamber, 31%; and postchamber, 25%. Using the method of generalized estimating equations with a logit link, we found no significant difference between the phases at a test level of $P = 0.05$. However, when comparing the postchamber and prechamber phases, we observed a P value of 0.069. This suggests the incidence of EBV shedding may actually be lower after chamber exposure, but because of the small number of subjects in the study, we were not able to reject the hypothesis of no difference at $P = 0.05$. Also, the EBV shedding patterns of the 60-day subjects and 91-day subjects were not significantly different.



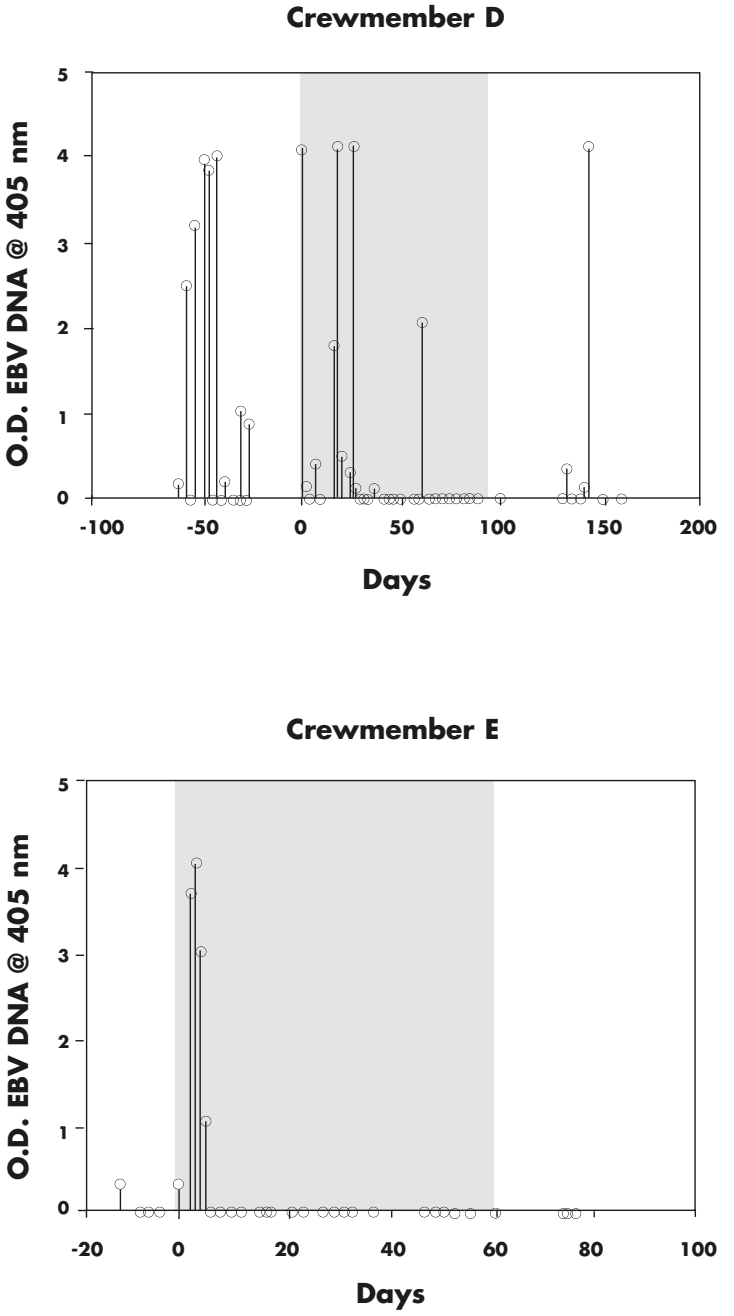
Shaded area represents in-chamber sampling period.
O.D.= optical density

Figure 5.3-1 *Individual EBV DNA shedding patterns for test subjects*



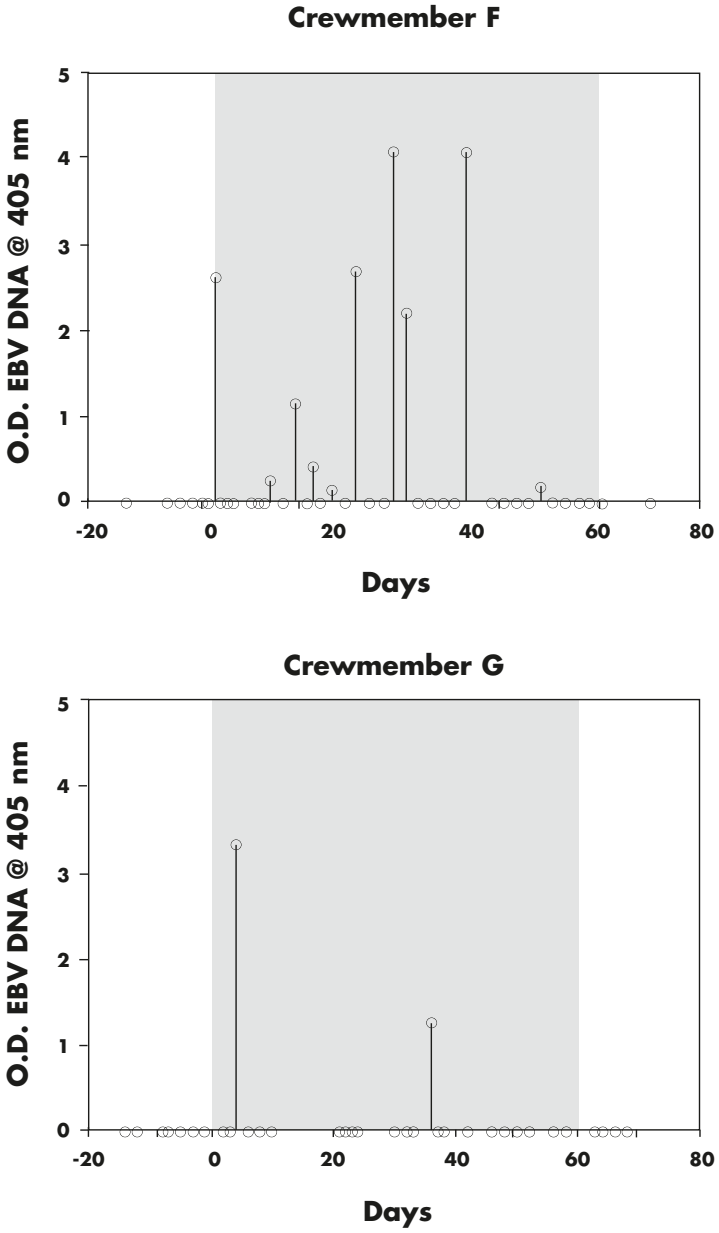
Shaded area represents in-chamber sampling period.
O.D.= optical density

Figure 5.3-1 continued *Individual EBV DNA shedding patterns for test subjects*



Shaded area represents in-chamber sampling period.
O.D.= optical density

Figure 5.3-1 continued *Individual EBV DNA shedding patterns for test subjects*



Shaded area represents in-chamber sampling period.
O.D.= optical density

Figure 5.3-1 continued *Individual EBV DNA shedding patterns for test subjects*

Sixteen urine samples collected from the subjects of both chamber studies before and after the in-chamber phase were analyzed for CMV DNA. Interestingly, not a single sample showed evidence of CMV DNA. The presence of CMV DNA in urine from the control group was also rare (1/81 samples).

Viral antibody titers for EBV and CMV, measured in samples from all 8 subjects before and after the study, are given in Table 5.3-3. Because viral antibody titers of subjects in the two studies were not significantly different, the data were pooled for further analysis. No significant differences in EBV VCA, EBV EA, or CMV IgG antibody titers were observed before and after either of the studies. EBV titers did not differ from those of the control group. CMV antibody titers of subjects were greater than those of the control group before and after the study.

Table 5.3-3 *Viral antibody titers before and after the 60-day and 91-day chamber studies*

Viral Antibody	Antibody Titer (Mean ± SE)				
	Chamber Study		Control Group		
	Pre-	Post-	Day 1	Day 7	Day 22
EBV VCA IgG[1]	7.32 ± 0.32	7.52 ± 0.34	6.12 ± 0.65	6.22 ± 0.52	6.12 ± 0.50
EBV EA IgG[2]	5.02 ± 0.41	4.44 ± 0.12	5.52 ± 0.31	5.96 ± 0.35	5.98 ± 0.32
CMV IgG[3]	6.82 ± 0.18[4]	6.94 ± 0.18[4]	3.09 ± 0.31	2.91 ± 0.31	2.91 ± 0.31

[1]Antibody for viral capsid antigen of Epstein-Barr virus
[2]Antibody for early antigen of Epstein-Barr virus
[3]Antibody for cytomegalovirus
[4]Statistically significant as compared to control ($P < 0.05$)

Discussion

This is the first study of EBV and CMV reactivation in subjects in a closed chamber, serving as a ground-based space analog. Current data show significant reactivation and shedding of EBV DNA by PCR occurred before, during, and after chamber isolation. With the exception of two subjects, the saliva specimens containing EBV DNA were 3 to 20 times higher than a healthy control group. Even though no CMV DNA was detected in urine by PCR, elevated CMV antibody titers indicated reactivation had occurred before isolation and perhaps continued during the isolation phase. This is consistent with stress being the initiator. These results are similar to our previous findings in Antarctic expeditioners (7) and astronauts (6, 8, 11, 14). However, some differences were found. For example, we frequently detected CMV in astronaut urine unlike the chamber studies. Also, progressively

increasing levels of antibodies to the viral capsid antigen of EBV were found in astronauts before, during, and after space flight (8, 14). No quantitation of viral DNA was conducted in the current chamber studies, whereas eight-fold increases in EBV DNA were observed in Space Shuttle crewmembers (6). Moreover, the current results in chamber subjects support and extend our previous observations that latent viral reactivation increased during space flight and Antarctic winter-over.

Recently, we demonstrated increased salivary shedding of EBV following diminished CMI response during the 8 to 9 months of isolation in the Antarctic (7). These findings are consistent with reductions in CMI response observed by Drs. Sams, D'Aunno, and Feeback during the 91-day chamber study (reported in Chapter 5.4 of this publication). Their results demonstrate a reduction in the ability of the subjects to respond to selected recall antigens, indicating a diminished CMI. Their findings are similar to our findings in Antarctic expeditioners (7).

SIGNIFICANCE

Space flight represents a unique environment for humans to work and live in, and astronauts experience numerous forms of stress from variable gravitational forces, isolation, confinement, and a variety of psychosocial factors. Stress associated with space flight results in increased levels of stress hormones and decreased cellular immunity, and now we have demonstrated increased EBV and CMV reactivation and shedding in astronauts and closed-chamber subjects. These findings are consistent with the stress model showing the effects of stress being mediated through the hypothalamus-pituitary-adrenal axis (3).

Future studies should be expanded to include behavioral assessment and study of selected stress hormones and additional latent viruses (e.g., human herpes virus 6, herpes simplex virus types 1 and 2, and varicella-zoster virus). Quantification of shed viruses will be included to determine if the number of shed viral copies increases during decreased CMI.

Based on the viral shedding and viral antibody response, the chamber isolation model serves as a good ground-based analog for space flight viral reactivation studies. Reduced cellular immunity and increased reactivation of EBV and CMV associated with chamber isolation are consistent with the Antarctic winter-over stress model and space flight experiences. The chamber isolation analog has proved to be a cost-effective model for studies of space flight-associated stress and the resulting cascade of human physiological effects.

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